

Gamma Interferon Triggers Interaction between ICSBP (IRF-8) and TEL, Recruiting the Histone Deacetylase HDAC3 to the Interferon-Responsive Element

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ICSBP (IRF-8) is a transcription factor of the IRF family expressed only in the immune system. It is induced in macrophages by gamma interferon (IFN- γ) and contributes to macrophage functions. By interacting with Ets family protein PU.1, ICSBP binds to the IRF/Ets composite element and stimulates transcription. ICSBP binds to another DNA element, the IFN-stimulated response element (ISRE), a common target of the IRF family. Limited knowledge as to how ICSBP and other IRF proteins regulate ISRE-dependent transcription in IFN- γ -activated macrophages is available. By mass-spectrometric analysis of ISRE-bound proteins in macrophages, we identified TEL, another Ets member, as a factor recruited to the element in an IFN- γ -dependent manner. In vitro analysis with recombinant proteins indicated that this recruitment is due to a direct interaction between ICSBP and TEL, which is enhanced by the presence of ISRE. Significantly, the interaction with TEL in turn resulted in the recruitment of the histone deacetylase HDAC3 to the ISRE, causing increased repression of IFN- γ -mediated reporter activity through the ISRE. This repression may provide a negative-feedback mechanism operating after the initial transcriptional activation by IFN- γ . By associating with two different Ets family proteins, ICSBP exerts a dual function in IFN- γ -dependent gene regulation in an immune system-specific manner.

ICSBP (IRF-8) is one of the nine members of the IRF family of transcription factors. Each member binds to the common target element, the interferon (IFN)-stimulated response element (ISRE), and regulates transcription of IFN-inducible genes as well as the IFN- α and - β genes (37, 50). Several members are potent activators of ISRE-dependent transcription. For example, IRF-9 and IRF-3, which are present in a latent form prior to stimulation, are activated by IFNs as well as viruses and are responsible for immediate activation of target genes. IRF-1, inducible after IFN and viral stimulation, also activates transcription. On the other hand, other members of the IRF family, the prototype being IRF-2, repress ISRE-dependent transcription. This repression is thought to contribute to the maintenance of IFN-inducible genes in a silent state prior to stimulation. Likewise, previous reporter analysis indicated that ICSBP is a repressor of ISRE-dependent transcription (36, 57).

Although the majority of IRF family members are expressed broadly in many cell types, ICSBP and another member, Pip (IRF-4), are expressed only in the immune system (4, 12, 49). Studies of ICSBP^{-/-} mice showed that ICSBP contributes to various aspects of innate and adaptive immunity (19, 43). In particular, this transcription factor has a nonredundant role in

the development and function of macrophages (48, 49, 52, 54). In macrophages ICSBP is strongly induced upon IFN- γ stimulation (22). Because IFN- γ is a macrophage-activating factor and is involved in many activities associated with activated macrophages (2), ICSBP is thought to have an important role in macrophage activation (49). Despite the functional implications, to date little information as to how ICSBP exerts its role at the level of transcription in activated macrophages is available. For example, it has not been quite clear whether repression of ISRE-mediated transcription by ICSBP, observed previously in nonimmune cells (36, 57), is relevant to activated macrophages. It has also been unclear whether activated macrophages have a unique mechanism of regulating ISRE-dependent transcription and, if so, what role ICSBP plays.

ICSBP and Pip regulate transcription from another target element, the EICE, a composite element consisting of the half site of the ISRE and the Ets site (4, 5, 38, 40). The binding of ICSBP to EICE depends on the interaction with PU.1, a member of the Ets family of transcription factors (44). PU.1 is expressed only in hematopoietic cells, and its expression pattern in adult mice is very similar to that of ICSBP (26). Analogous to the PU.1-Pip interaction, the PU.1-ICSBP interaction is DNA directed and facilitated by EICE (4, 38). The PU.1-ICSBP complex and the PU.1-Pip complex are shown to act as a stimulators of EICE-mediated transcription (4, 40). Recent studies indicate that the EICE and EICE-like elements are present in a number of IFN- γ -stimulated genes active in macrophages (13, 23, 25, 31, 42) and that ICSBP and PU.1 cooperatively stimulate transcription of some of these genes. Acti-

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vation of EICE-dependent transcription appears to involve phosphorylation of PU.1 as well as ICSBP (28, 45). This activation may in part be due to the recruitment of coactivator/histone acetylase CBP/p300, since CBP/p300 can interact with PU.1 (but not ICSBP) (32, 59).

TEL is another member of the Ets family, originally discovered as part of the TEL/platelet-derived growth factor receptor β fusion protein in chronic myelomonocytic leukemia (15, 21). Later, TEL was shown to fuse to a number of other proteins in other leukemias (16, 18). It has been shown that, through a differential ATG usage, a single *tel* gene generates at least two nuclear phosphoproteins (16, 39). Although TEL is expressed ubiquitously, a recent analysis with TEL^{-/-} embryonic stem cells indicates that it is specifically required for bone marrow hematopoiesis (56). TEL has a classic Ets domain in the C terminus and binds to the Ets site, with a core motif of GGAA/T (44). It also has the oligomerization domain (also called the pointed [PNT] domain, B domain, or HLH domain) in the N terminus that is conserved in several Ets members, but not in PU.1 (33, 39). TEL represses transcription through the Ets site, and repression may be attributed to the recruitment of mSin3A, corepressor N-CoR, and histone deacetylase HDAC3 (7, 14, 29, 33, 55).

The present study was carried out with the aim of understanding the function of ICSBP in ISRE-dependent transcription in a biologically relevant system, i.e., IFN- γ -activated macrophages. We surmised that, if ICSBP has a unique mechanism of regulating ISRE-mediated transcription in macrophages, then such a mechanism may be reflected in protein complexes bound to the ISRE. With this idea in mind, we sought to identify factors that bind to the ISRE along with ICSBP in IFN- γ -stimulated macrophages. The initial mass-spectrometric analysis identified TEL as a factor recruited to the ISRE upon IFN- γ stimulation. Subsequent in vitro studies demonstrated that it is ICSBP that recruits TEL to the ISRE. Detailed deletion analysis revealed that domains within TEL and ICSBP required for the interaction differ from those responsible for the interaction with PU.1. By cotransfection assays we found that the combination of TEL and ICSBP strongly represses the activities of ISRE reporters as well as endogenous gene expression upon IFN- γ addition, indicating the functional significance of TEL recruitment. Finally, we show that recruitment of TEL to the ISRE in turn brings the histone deacetylase HDAC3 to the element, a likely mechanism of increased repression by TEL. Taken together, the results of this study show that ICSBP plays a dual role in activated macrophages by interacting with two different Ets family proteins.

MATERIALS AND METHODS

Cells and plasmids. Raw 264.7 (RAW) cells were maintained in RPMI1640 (Quality Bioproducts) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and penicillin-streptomycin (Life Technologies) at 37°C under 5% CO₂. Cells were treated with recombinant IFN- γ (100 U/ml) or natural murine IFN- α/β (1,000 U/ml) for 12 h (32). Glutathione S-transferase (GST) fusions with full-length TEL and TEL deletion mutants were constructed by inserting the following PCR fragments into pGEX-4T (Pharmacia). The C-terminal deletion mutants 1-419 and 1-332 were produced with primers corresponding to the in-frame *SaI* site at the 5' end and the deletion points. The Δ Ets fragment was prepared by blunt-end ligation of two fragments (1-332 and 420-452). Δ PNT, Δ 55-332, Δ 55-245, and Δ 55-419 fragments were prepared by ligating the 1-54 fragment and corresponding C-terminal fragments. The information on primer sequences and PCR conditions is available upon request. All reactions were

performed with *Pfu* DNA polymerase (Stratagene), the resultant constructs were sequenced, and correct deletions were confirmed. The mammalian expression vector for full-length human TEL (hTEL) cDNA (pcDNA-hTEL) has been described (15). Vectors for TEL deletion mutants were constructed by inserting appropriate fragments in the pcDNA vector. The pcx-ICSBP vector has been described (54). Clones of 32D myeloid cells stably transfected with full-length hTEL or an empty vector are described elsewhere (R. Martinez et al., submitted for publication). These cells were transduced with a retrovirus vector containing ICSBP, pMSCVpuro-ICSBP, as described previously (48).

Antibodies. Antibodies against IRF-1, IRF-2, and ICSBP have been described (32). A polyclonal rabbit antibody against TEL was produced with the peptide corresponding to a carboxy-terminal region of TEL (C-DRLEHLESQELDEQ IYQEDE) as an immunogen. The sera were affinity purified with the peptide by using the SulfoLink kit (Pierce). The antibodies against HDAC1, HDAC2, and HDAC3 were obtained from Santa Cruz Biotechnology.

DNA affinity binding assay. Nuclear extracts were prepared as described previously (11) with some modifications. Briefly, 4×10^8 cells were homogenized in 3 ml of buffer A, and nuclear pellets were suspended in 1.2 ml of buffer C. Eight hundred microliters of nuclear extracts (~1 mg of proteins) was mixed with 160 μ l of 50% glycerol and 1 μ l of 10% NP-40. Affinity binding assays were performed principally as described previously (32). Briefly, three repeats of the ISRE from the guanylate binding protein (GBP) gene (10, 53) were amplified by PCR with a biotinylated 5' primer (GAGGTACCGAGCTCTTAC) and a 3' primer (ACCAACGTCGCGAGATCT). Mutant ISRE oligonucleotides with *XhoI* and *BglII* sites at the 5' and 3' ends, respectively, were synthesized and inserted into the pGL-L₄₀Luc plasmid. Amplified DNA was conjugated to M280 magnetic beads (Dynal) and incubated with ~1 mg of nuclear extracts in the presence of 20 μ g of salmon sperm DNA for 1 h at 4°C. Beads were washed three times with washing buffer (20 mM Tris [pH 7.5], 0.2 mM EDTA, 300 mM NaCl, 10% glycerol, 0.01% NP-40), and bound materials were eluted in 1 \times sodium dodecyl sulfate (SDS) sample buffer. Samples were separated by SDS-8% polyacrylamide gel electrophoresis (PAGE). Gels were visualized with silver staining or colloidal Coomassie blue staining (Sigma). Sliced bands were subjected to mass spectrometry. For immunoblot detection, 100 to 200 μ g of nuclear extracts was incubated with ISRE-conjugated beads, and bound materials were separated by SDS-10% PAGE (Novex). Recombinant, histidine-tagged IRF-1, IRF-2, and ICSBP have been described (32). Recombinant, histidine-tagged TEL was constructed with a baculovirus vector with full-length hTEL cDNA (15) by using the Bac-to-BAC system (Life Technologies). Recombinant TEL, IRF-1, IRF-2, and ICSBP proteins were affinity purified on Ni²⁺-nitrilotriacetic acid resin (Qiagen) as described previously (32). Twenty to 100 ng of recombinant proteins was incubated with ISRE-conjugated beads for 1 h at 4°C. For immunoblotting, gels were transferred onto a nitrocellulose membrane (Schleicher & Schuell) with a wet transfer apparatus (Bio-Rad). Membranes were blocked with 5% milk and incubated with a primary antibody followed by incubation with a horseradish peroxidase-conjugated anti-rabbit antibody (Amersham) and developed by the chemiluminescence method (Pierce).

In gel digestion and tandem mass spectrometry. The Coomassie blue-stained bands were excised and destained with 30% methanol for 3 h at room temperature and washed twice for 30 min with 150 μ l of 1:1 acetonitrile-0.1 M ammonium bicarbonate, pH 8. Trypsin digestion and mass-spectrometric sequencing of proteins from polyacrylamide gels were carried out essentially as described previously (46). Briefly, gel slices were diced into 1-mm cubes and rehydrated in 10 μ l of 0.1 M ammonium bicarbonate buffer containing 0.5 μ g of sequencing grade modified trypsin (Promega). An additional 15 μ l of digestion buffer without trypsin was added after 10 min. Digestion was carried out for 16 h at 37°C. Peptides were extracted with 150 μ l of 60% acetonitrile containing 0.1% trifluoroacetic acid at 30°C for 30 min and concentrated to 15 μ l with an Eppendorf Speed-Vac concentrator. A Finnigan-MAT LCQ ion trap mass spectrometer equipped with electrospray interface and a reverse-phase (RP) microcapillary high-performance liquid chromatography column (interior diameter, 75 μ m; packed with 10 cm of 5- μ m C18 RP particles) was used to collect the collision-induced dissociation spectra. About 2 μ l of the peptide digest was used for the analysis. The instrument was operated in positive polarity mode and was set to collect a full scan (m/z 250 to 2,000) and a data-dependent MS/MS on the most intense ion. Collision-induced dissociation spectra were interpreted by using either the SEQUEST program (ThermoFinnigan) or Protein Prospector (www.prospector.ucsf.edu).

GST pull-down assays. GST fusions to TEL and TEL deletion mutants (1 μ g) were conjugated to 20 μ l of 50% glutathione-Sepharose beads (Pharmacia) at 4°C for 1 h. [³⁵S]methionine-labeled full-length ICSBP and ICSBP deletion mutants (48) were prepared by an in vitro transcription and translation kit (Promega). Control GST and GST fusions conjugated to the beads were incu-

bated with 20 μ l of labeled materials at 4°C for 1 h in 1 ml of binding buffer and washed with binding buffer, and bound materials were eluted in 1% SDS sample buffer and detected by autoradiography.

Reporter analysis. The luciferase reporter containing three tandem repeats of ISRE from the GBP gene and the minimum basal promoter, pGL2-(ISRE)3L₄₀-Luc, has been described (53). This reporter (0.4 μ g) was transfected into 2.5×10^5 RAW cells in 12-well plates along with 0.4 μ g of pcx-ICSBP and/or 0.4 μ g of pcDNA-TEL and 5 ng of the *Renilla* plasmid used as an internal control. Cells were treated with IFN- γ (100 U/ml) for 12 h prior to harvest. Luciferase activity was measured by the dual luciferase assay kit (Promega) and normalized by the internal *Renilla* control. Expression of the wild-type TEL and TEL deletion mutants of the expected sizes was confirmed by immunoblot analysis of whole-cell lysates from transfected cells.

Reverse transcription and real-time reverse transcription-PCR. 2',5'-oligoadenylate synthetase (2',5'-OAS) transcripts were measured by the real-time fluorescence detection method using an ABI PRISM 7700 sequence detection system (Applied Biosystems). One microgram of total RNA from 32D cells was reverse transcribed by using Superscript II Rnase H reverse transcriptase (Life Technologies). PCRs were performed with the SYBR green PCR master kit (Perkin-Elmer). The primer set for 2',5'-OAS (forward and reverse) was 5'-CTACCTGCTTCACGGAGCTC-3' and 5'-CTCCTTACACAGTTGGTACCAG-3', and that for hypoxanthine phosphoribosyltransferase (HPRT) was 5'-GGGAGGCCATCACATTGTG-3' and 5'-TCCAGCAGGTCAGCAAAGAAC-3', respectively. SYBR green was incorporated into the reaction mixture to permit product measurement. Each of the primer sets gave a unique product. Real-time PCR values were determined by reference to a standard curve generated by a serial dilution of cDNA. Values obtained for 2',5'-OAS mRNAs were normalized by the levels of HPRT mRNA.

RESULTS

Mass-spectrometric identification of TEL in the ISRE-bound protein complexes. In an effort to identify proteins that are recruited to the ISRE in macrophages following IFN- γ treatment, we employed DNA affinity binding assays. Three copies of the ISRE from the GBP gene (6, 10) immobilized onto magnetic beads were incubated with nuclear extracts from RAW cells treated with IFN- γ for 12 h. RAW cells are a murine cell line with macrophage functions and are activated by IFN- γ (22, 54). As a control, a mutant ISRE containing a A \rightarrow C mutation in the both halves of the GAAA motif that abolished IRF binding activity was tested in parallel (see the sequence for the wild-type and mutant ISRE in Fig. 1A, bottom). Bound materials were eluted, separated by SDS-8% PAGE, and visualized by silver staining. As seen in Fig. 1A, extracts from untreated cells revealed multiple bands ranging from ~97 to 55 kDa that were also present in IFN- γ -treated samples. Importantly, extracts from IFN- γ -treated cells revealed two additional bands of ~62 and ~58 kDa that were not present in untreated cells (bands 1 and 2, Fig. 1A). Although present prior to IFN- γ treatment, bands 3 and 4 increased in intensity following IFN- γ treatment. These bands represented proteins specifically assembled on the ISRE, since the mutant ISRE did not reveal any of these bands. To determine the identities of these proteins, bands 1, 2, and 3 were excised from gels prepared in a larger scale, stained with Coomassie blue, and digested with trypsin. Digested materials were separated by microcapillary high-performance liquid chromatography and analyzed on-line by ion trap mass spectrometry (1). As summarized in Table 1, five and four peptides in bands 1 and 2, respectively, corresponded to peptide sequences of the murine TEL (15, 39). The murine *tel* gene, like the human counterpart, produces at least two species of peptides translated from distinct ATG sites (39). Band 1 and band 2 likely represent these two products. Five peptides from band 3 were identified

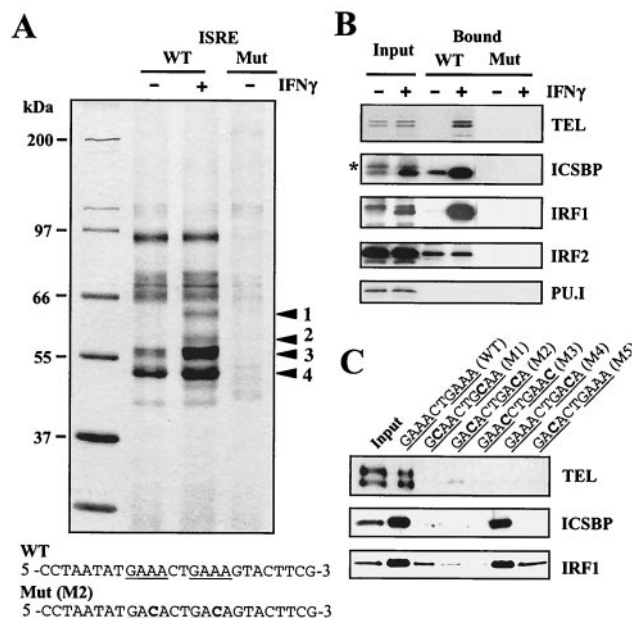


FIG. 1. IFN- γ -dependent recruitment of TEL to the ISRE. (A) Three copies of the wild-type (WT) and mutant (Mut) ISRE immobilized to magnetic beads were incubated with nuclear extracts from RAW cells treated with or without IFN- γ (100 U/ml) for 12 h. Bound materials were separated by SDS-8% PAGE and visualized by silver staining. Bands 1 and 2 and band 3 (arrowheads) were identified as TEL and ICSBP, respectively (Table 1). (B) Immobilized wild-type ISREs were incubated with 100 μ g of extracts from RAW cells treated with IFN- γ for 12 h, and bound proteins were separated by SDS-10% PAGE and detected by immunoblotting. Input represents 5% of total extracts. *, nonspecific protein that did not bind to the ISRE. (C) The wild-type and mutant ISREs were immobilized to the beads, and the binding of the indicated factors was tested by immunoblotting as for panel B. Input represents 5% of total extracts.

as arising from the mouse ICSBP protein. Band 4 was determined to be IRF-1 by immunoblot analysis (see below).

To confirm the results of mass-spectrometric analysis, bound proteins were analyzed by immunoblotting using an antibody raised against a peptide of 20 amino acids present in the C-terminal region of the murine TEL. As shown in the input lanes in Fig. 1B, the antibody recognized two species of TEL in RAW cell extracts that were present in similar amounts before and after IFN- γ treatment, indicating that TEL expression levels did not alter following IFN- γ treatment. The two bands correspond to band 1 and band 2 in Fig. 1A. In contrast, levels of ICSBP and IRF-1 were enhanced after IFN- γ treatment (compare - and + input lanes in Fig. 1B), consistent with IFN- γ induction of the IRF proteins (22, 53). As seen in the bound lanes in Fig. 1B, neither TEL species bound to the ISRE in untreated samples. However, both of the TEL species bound to the ISRE in samples treated with IFN- γ . Supporting the specificity of binding, TEL was not found on the mutant ISRE, regardless of IFN- γ treatment. Another peptide antibody raised against an internal region of TEL produced the same results (not shown). Also as shown in Fig. 1B, the amounts of ICSBP and IRF-1 bound to the ISRE were markedly increased after IFN- γ treatment, consistent with the increased expression. An upper band in the ICSBP immunoblot was a nonspecific protein and did not bind to the ISRE. In addition, IRF-2,

TABLE 1. Peptides identified by mass spectrometry

Band	Accession no.	Protein	Sequences ^a
1	3023730 (NCBI nr)	TEL	SSTPLHVHTVPR (aa 29–40), MEEDSIHLPTHLR (aa 44–56), LQPIYWSR (aa 57–64), DDVAQWLK (aa 65–72), GNLPTGTAGGVMEAGELGVAVK (aa 457–478)
2	3023730 (NCBI nr)	TEL	LNIR (aa 400–404), ALLLTK (aa 94–100), DDVAQWLK (aa 65–72), LQPIYWSR (aa 57–64)
3	P23611 (Swiss-Prot)	ICSBP	QDYNQEVDASIFK (aa 45–58), SPDFEEVTDR (aa 89–98), SQLDISEPYK (aa 99–108), LSLSQPLPK (aa 229–238), LILVQVEDQLYAR (aa 362–373)

^a Peptide sequences identified by mass spectrometry. The numbers in parentheses indicate amino acid (aa) positions within the murine TEL (39) and ICSBP (12).

expressed before and after IFN- γ treatment at similar levels, bound to the ISRE. The binding of multiple IRF members to the ISRE may be attributable to the fact that ICSBP interacts with IRF-1 and IRF-2, conferring on ICSBP an increased affinity for the DNA (3, 34). Because the recruitment of TEL was unexpected and because another Ets member, PU.1, interacts with ICSBP to bind to EICE (5, 26, 30, 48), we next examined whether PU.1 is also recruited to the ISRE. As shown in Fig. 1B, this Ets member did not bind to the ISRE, irrespective of IFN- γ treatment.

To further investigate the nucleotides in the ISRE important for TEL recruitment, additional mutants shown in Fig. 1C were tested in DNA affinity binding assays with IFN- γ -treated RAW cells. M1, M2, and M3 each had a point mutation in both halves of the GAAA repeat, whereas M4 and M5 each had a mutation in only the 5' or 3' half of the repeat. As shown in Fig. 1C mutations of both halves of the GAAA greatly diminished the binding of ICSBP and IRF-1 and completely abolished TEL recruitment. Interestingly, M4, with a mutation in the 3' half of the repeat and retaining an intact 5' GAAA, permitted the binding of both ICSBP and IRF-1. However, TEL recruitment was completely eliminated with this mutation. On the other hand, M5, with a mutation in the 5' half and an intact 3' GAAA, did not permit the binding of ICSBP or the recruitment of TEL. IRF-1 binding was seen with this mutant, albeit at a reduced level. These results indicate that TEL recruitment is dependent on the presence of the intact GAAA motifs in both halves of the ISRE and that, even when ICSBP and IRF-1 are bound to the ISRE, TEL cannot be recruited to the element if the element has a mutation in the 3' half of the repeat. This implies that TEL has some affinity for the 3' GAAA and that ICSBP and TEL bind to the element with a defined orientation. Taken together, the results of this analysis identify TEL as a factor recruited to the ISRE upon IFN- γ treatment.

TEL recruitment requires ICSBP. To investigate the mechanism by which TEL is recruited to the ISRE, a baculovirus recombinant protein was prepared from full-length hTEL (15) and its binding to the ISRE was tested in vitro along with that of recombinant ICSBP, IRF-1, and IRF-2. The hTEL is ~90% identical to murine TEL, although the latter has an additional 37 amino acids in the C terminus (39). In Fig. 2A, the immobilized ISRE was incubated with the indicated recombinant proteins and bound proteins were eluted and detected by immunoblotting. As expected, TEL alone did not bind to the ISRE. However, TEL was bound to the ISRE when coincubated with ICSBP. ICSBP by itself bound only weakly to the ISRE, as previously noted (34, 48), but coincubation with TEL

increased the binding, as evidenced by increased intensity of the band. Significantly, TEL did not bind to the ISRE when coincubated with IRF-1 or IRF-2. In Fig. 2A (right), TEL binding in the presence of several IRF proteins was further tested. TEL did not bind to the element when coincubated with IRF-1 plus IRF-2 but bound well when incubated with ICSBP plus IRF-1 or IRF-2. TEL binding was also detected when TEL was coincubated with all three recombinant proteins. In these experiments TEL binding appeared somewhat weaker when TEL was incubated in the presence of IRF-2 than when it was incubated in the presence of IRF-1. These results indicate that TEL does not bind to the ISRE by itself but binds to it in the presence of ICSBP, suggesting that recruitment of TEL to the ISRE is dependent on ICSBP. To assess how ICSBP recruits TEL to the ISRE, an order-of-addition experiment was performed; in this experiment TEL or ICSBP was added to the immobilized DNA alone, unbound materials were washed, and then the second protein was added. As seen in

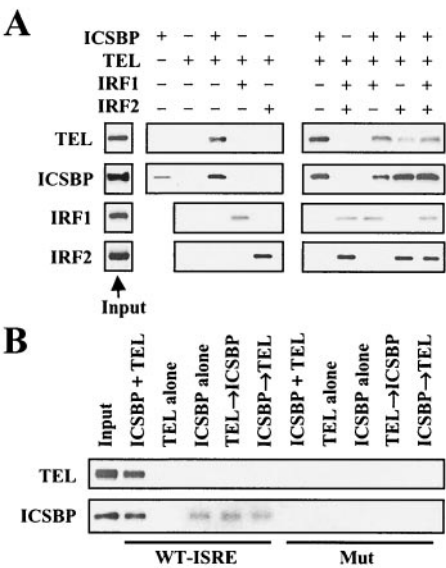


FIG. 2. ICSBP-dependent recruitment of recombinant TEL to the ISRE. (A) Immobilized wild-type ISREs were incubated with 20 ng of the indicated recombinant proteins for 1 h at 4°C, and bound proteins were detected by immunoblot analysis. Input represents 10% of the total reaction mixture. (B) Immobilized wild-type (WT) and mutant (Mut) ISREs (M2) were first incubated with 50 ng of the indicated recombinant proteins for 1 h at 4°C, washed, and then incubated with the second protein under the same conditions. Bound proteins were detected as for panel A. Input represents 5% of total extracts.

Fig. 2B, TEL was not recruited to the ISRE when added alone prior to the addition of ICSBP. Likewise TEL was not recruited when ICSBP was added before TEL. In contrast, TEL binding was observed when the two proteins were coincubated and added to the ISRE at the same time. However, when TEL and ICSBP were coincubated and added to the mutant ISRE (M2), neither protein bound to the DNA (Fig. 2B), verifying the target specificity of binding. These results indicate that TEL and ICSBP directly interact with each other to bind to the ISRE.

It is of note here that the standard electrophoretic gel mobility shift assays (EMSA) did not reveal the binding of recombinant TEL to ISRE probes in the presence or absence of ICSBP. TEL binding was not detected in EMSA with RAW cell extracts either (not shown), suggesting that TEL associates with the ISRE with a relatively low affinity and dissociates from the complex during electrophoresis. This property differs from that of the PU.1-ICSBP interaction, which is readily seen by EMSA with the EICE probe (4, 5, 38, 40) (see Discussion).

Domain analysis. To determine the domains within TEL that are required for interacting with ICSBP, a series of GST-TEL fusions were constructed and tested for binding of [³⁵S]methionine-labeled ICSBP. Figure 3A, top, shows a diagram of the GST-TEL deletion mutants and Coomassie blue staining of them. Figure 3A, bottom, shows the binding of radiolabeled ICSBP. In agreement with DNA affinity binding data in Fig. 2, ICSBP bound to the GST fusion to full-length TEL (WT). In contrast, it did not bind to the C-terminal deletion mutants, 1-245, 1-332, and 1-419, or to ΔEts, indicating that both the extreme C-terminal domain and the Ets domain are required for interacting with ICSBP. On the other hand, ICSBP binding was not affected by the deletion of the pointed domain (ΔPNT), involved in oligomerization (16, 21). Similarly, ICSBP bound to Δ55-245 and Δ55-332, which lack the internal region, although binding to the latter was weaker than binding to the former. These results indicate that regions encompassing the extreme C-terminal domain and the Ets domain of TEL are required for the interaction with ICSBP. This domain requirement differs somewhat from that of PU.1, which requires, in addition to the Ets domain, the PEST domain, N-terminal from the Ets domain (5, 38).

To study domains within ICSBP that are required for binding to TEL, a GST fusion to full-length TEL and control GST were tested for binding to a series of radiolabeled ICSBP deletion mutants. Figure 3B, top, shows a diagram of ICSBP deletion mutants. Full-length ICSBP and all C-terminal deletion mutants, 1-390, 1-356, 1-305, and 1-254, bound to TEL but not control GST. Moreover, the mutant that contains only the DNA binding domain (DBD) bound to TEL. In contrast, the mutant with a deletion of the DBD (ΔDBD) failed to bind to TEL. Interestingly, K79E, containing a point mutation in the DBD known to be critical for ISRE binding, failed to bind to TEL. These data indicate that ICSBP interacts with TEL through the DBD. This domain requirement differs from that of the interaction with PU.1, which is dependent on the IAD (IRF association domain) region in the C-terminal domain (5, 48).

TEL represses ISRE-dependent transcription. To investigate the functional significance of TEL recruitment, transient transfection assays were carried out using a luciferase reporter driven by three copies of the ISRE identical to that used above.

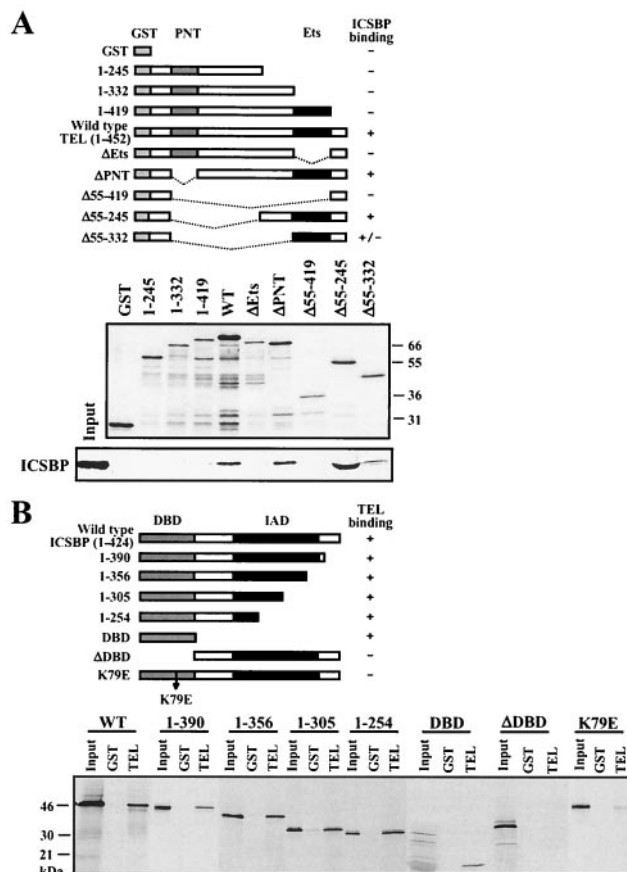


FIG. 3. Domains required for TEL-ICSBP interaction. (A) (Top) Diagram of TEL deletion mutants and summary of ICSBP binding. (Bottom) Coomassie blue staining of GST-TEL fusions and binding of ³⁵S-labeled ICSBP. Input represents 10% of total reaction. WT, wild type. (B) (Top) Diagram of ICSBP deletion mutants and summary of TEL binding. (Bottom) ³⁵S-labeled ICSBP deletion mutants were incubated with control GST or GST-full-length TEL, and bound materials were detected by autoradiography. Input represents 10% of the reaction mixture.

RAW cells were transfected with the reporter along with expression vector for ICSBP, TEL, or both and were treated with or without IFN- γ for 12 h. As seen in Fig. 4A, transfection of ICSBP alone reduced ISRE reporter activity by ~40% irrespective of IFN- γ treatment, consistent with the previous finding that ICSBP represses ISRE-dependent transcription (36, 57). On the other hand, transfection of TEL alone had little effect on reporter activity, also in the presence or absence of IFN- γ . When ICSBP and TEL were cotransfected, reporter activity was further reduced by ~70%, again irrespective of IFN- γ treatment. Under these conditions ISRE reporter activity was only modestly stimulated by IFN- γ , which may be due to the repression caused by the endogenous ICSBP and TEL expressed in RAW cells. These results indicate that ICSBP and TEL cooperatively repress ISRE-dependent reporter activity. Similar cooperative repression was seen when cotransfection experiments were performed with P19 cells, which do not express endogenous ICSBP (not shown).

To examine whether TEL deletion mutants that failed to interact with ICSBP also failed to repress ISRE-mediated tran-

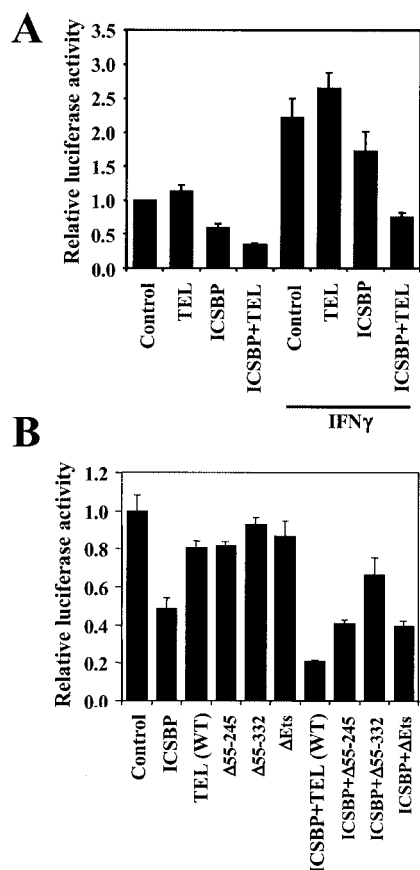


FIG. 4. Increased repression of ISRE-reporter activity by TEL and ICSBP. (A) RAW cells were transfected with 0.4 μ g of ISRE-luciferase reporter along with 0.4 μ g of pcx-ICSBP and/or pcDNA-TEL and the *Renilla* plasmid (5 ng) for 12 h. The total amount of transfected DNA was adjusted with appropriate empty vector to 1.205 μ g. Cells were then treated with or without IFN- γ for 12 h. Luciferase activity was normalized by the *Renilla* internal control. Values represent the averages of three determinations \pm standard deviations (SD). (B) RAW cells were transfected with ISRE-reporter along with pcx-ICSBP and the indicated TEL deletion mutants and the *Renilla* control as for panel A. Cells were treated with IFN- γ for 12 h, and luciferase activity was measured as for panel A. Values represent the averages of three determinations \pm SD.

scription, RAW cells were transfected with the reporter along with TEL deletion mutants and the wild-type ICSBP and reporter activity was tested following IFN- γ treatment. Deletion mutants Δ 55-245 and Δ 55-332 retain the ability to interact with ICSBP, while deletion mutant Δ Ets does not interact with ICSBP (Fig. 3A). Expression of the wild-type TEL and the TEL deletion mutants of the expected sizes was detected by immunoblot analysis of transfected cells (not shown). Results of reporter analysis are shown in Fig. 4B. As expected, wild-type TEL, when cotransfected with ICSBP, repressed reporter activity by \sim 65% but did not repress reporter activity when transfected alone. In contrast, none of the four TEL deletion mutants tested repressed reporter activity to a level similar to that by the wild-type TEL, irrespective of whether they retained ICSBP binding activity. The reporter activities obtained with Δ 55-245 and Δ Ets did not significantly differ from those

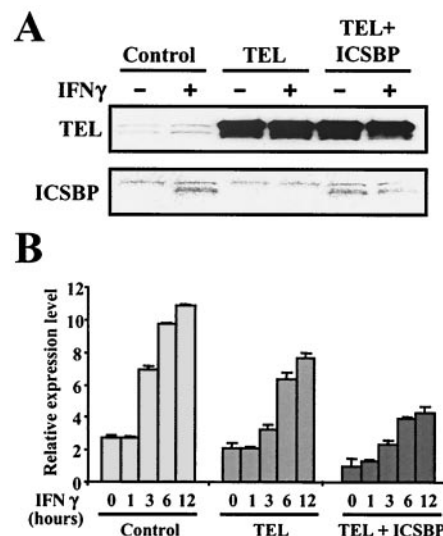


FIG. 5. Reduced 2',5'-OAS transcript expression by TEL and ICSBP. (A) 32D cells were stably transfected with the control vector or full-length TEL, and appropriate clones were isolated. The TEL clone was transduced with control (TEL) or ICSBP retrovirus vector (TEL + ICSBP). Cells were treated with IFN- γ (100 U/ml) for 6 h. Expression of TEL and ICSBP proteins was detected by immunoblot analysis using 100 μ g of nuclear extracts. (B) 2',5'-OAS mRNA levels in indicated 32D clones treated with IFN- γ (100 U/ml) for the indicated periods were measured by real-time reverse transcription-PCR. Values represent relative mRNA levels normalized by HPRT.

with ICSBP alone, indicating that these deletion mutants did not have a repressive activity. The reporter activity observed with Δ 55-332 was slightly higher than those due to ICSBP alone, suggesting that this deletion might have interfered with the activity of endogenous TEL. These data indicate that repression requires broad regions of TEL beyond what is required for binding to ICSBP. Although there are other explanations for these results, it is possible that the repression is dependent on proteins other than ICSBP that interact with TEL (see below).

Ectopic expression of TEL and ICSBP represses IFN- γ -inducible 2', 5'-OAS gene expression. Next, we wished to ascertain whether TEL and ICSBP affect expression of an endogenous gene. To this end, we examined expression of the 2',5'-OAS gene in 32D cells that ectopically express TEL and ICSBP. The 2',5'-OAS gene (35, 41) is a classic IFN-inducible gene, and the promoter of this gene carries an ISRE (8) but not other elements, such as the IFN- γ -activated site, that might obscure the effect of TEL and ICSBP. 32D cells are a murine myeloid progenitor cell line where expression of endogenous ICSBP and TEL is low. The 32D cell clone stably transfected with TEL expressed a high level of TEL compared with control 32D cells. To achieve ectopic ICSBP expression in this clone, cells were transduced with a retrovirus vector for ICSBP (Fig. 5A, TEL + ICSBP) (48). As a control for viral transduction, the control clone and TEL-overexpressing clone were also transduced with a control virus without insert (Fig. 5A, control and TEL, respectively) and treated with IFN- γ for 12 h. Immunoblot assays in Fig. 5A show that TEL is overexpressed in the TEL clone while it is almost undetectable in the control clone. ICSBP was also essentially undetectable in the control

clone but was induced at a modest level by IFN- γ . However, the TEL clone did not induce ICSBP after IFN- γ addition, suggesting that IFN- γ induction of ICSBP is repressed by TEL. The absence of ICSBP expression is not due to the generalized lack of IFN- γ responsiveness, as verified by the analysis of genome-wide gene expression patterns in this clone (R. Martinez et al., submitted). On the other hand, ICSBP was constitutively expressed in the TEL-plus-ICSBP clone, and the expression was not affected by IFN- γ treatment, as expected. The level of ICSBP was similar to that seen in the control clone induced by IFN- γ . Data in Fig. 5B show levels of 2',5'-OAS transcripts in these clones as measured by real-time PCR. 2',5'-OAS mRNA levels were normalized by control HPRT mRNA levels. In the control clone, 2',5'-OAS expression increased linearly beginning 3 h after IFN- γ treatment and reached approximately fourfold by 12 h. Transcript levels in the TEL clone were also increased upon IFN- γ treatment, with kinetics similar to those for the control cells. However, the maximum transcript level was lower than that for the control clone, indicating that this clone is capable of inducing 2',5'-OAS after IFN- γ treatment, although less efficiently than the control clone. In contrast, the clone expressing both TEL and ICSBP expressed much lower levels of 2',5'-OAS transcripts both before and after IFN- γ treatment. In line with the low basal expression, IFN- γ led to a meager transcript induction, the levels reaching only ~35% of those in the control clone. Thus, the combination of TEL and ICSBP represses constitutive and IFN- γ -inducible expression of the 2',5'-OAS gene.

TEL recruits the histone deacetylase HDAC3 to the ISRE in an IFN- γ -specific manner. The reporter data shown in Fig. 4B indicate that TEL repression of ISRE-mediated transcription requires domains other than those required for ICSBP binding. This result raised the possibility that TEL interacts with other proteins important for transcriptional repression. A number of DNA binding transcription factors repress transcription by recruiting histone deacetylases to the promoter (9, 24). Indeed, TEL interacts with HDAC3, and this interaction is thought to account for transcriptional repression through the Ets site (55). TEL also interacts with mSin3A as well as SMRT and N-CoR (7, 14). The last three factors are part of the large histone deacetylase complexes (20, 51, 60). HDAC3 is a member of the class I histone deacetylases, which include HDAC1 and HDAC2. In many cell types HDAC1 and HDAC2 are more abundant than HDAC3 (9, 20, 24). Unlike the well-documented interaction of TEL with HDAC3, we have not obtained convincing evidence for direct interaction between ICSBP and class I histone deacetylases (not shown). In view of these observations, it was of interest to study whether histone deacetylases are recruited to the ISRE by TEL. Nuclear extracts from RAW cells treated with or without IFN- γ were incubated with the immobilized ISRE, and the bound materials were tested for the presence of histone deacetylases by immunoblot assays. As seen in Fig. 6A, HDAC1, HDAC2, and HDAC3 were all expressed in RAW cells and their levels remained similar before and after IFN- γ treatment (see the input lanes). TEL bound to the ISRE along with ICSBP in an IFN- γ -dependent manner, confirming the data in Fig. 1B. In addition, HDAC3 was recruited to the ISRE in an IFN- γ -dependent manner. However, neither HDAC1 nor HDAC2 was detected on the ISRE. Supporting the specificity of

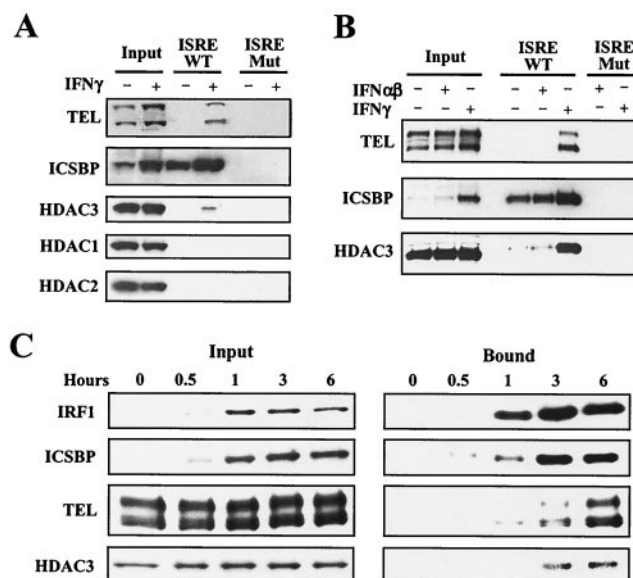


FIG. 6. IFN- γ -dependent recruitment of HDAC3 to the ISRE. (A and B) The immobilized wild-type (WT) and mutant (Mut) ISREs were incubated with 100 μ g of extracts from RAW cells treated with IFN- γ (100 U/ml) (A and B) or IFN- α/β (1,000 U/ml) (B) for 12 h, and bound proteins were detected by immunoblotting. Input represents 5% of total extracts. (C) Binding of indicated factors was tested with 100 μ g of extracts from RAW cells treated for the indicated times. Input represents 10% of total extracts.

HDAC3 recruitment, neither HDAC3 nor TEL bound to the mutant ISRE. These results indicate that HDAC3 is recruited to the ISRE upon IFN- γ treatment.

Since IFN- γ signals through a pathway partly shared with that of IFN- α/β and since the two cytokines regulate overlapping sets of target genes (47), we sought to determine whether IFN- α/β treatment also triggers recruitment of TEL and HDAC3 to the ISRE. As seen in Fig. 6B, TEL was not recruited to the ISRE following IFN- α/β treatment, although ICSBP, IRF-1, and IRF-2 bound to the ISRE. Furthermore, IFN- α/β led to little HDAC3 recruitment to the element. These results indicate that HDAC3 is recruited to the ISRE through TEL specifically following IFN- γ treatment.

Finally, it was of importance to begin addressing the biological significance of TEL recruitment, particularly because the assembly of the HDAC3 complex and the ensuing transcriptional repression from the ISRE appeared somewhat incompatible with the fact that IFN- γ activates ISRE-mediated transcription (6). We surmised that this transcriptional repression might be a secondary event that occurs following the initial activation by IFN- γ , since ICSBP is induced after IFN- γ treatment and since it takes time for the protein to accumulate in macrophages (22). To address this possibility, we examined the kinetics of ICSBP and TEL binding after various periods of IFN- γ treatment. IRF-1 was included in our tests, because IFN- γ activation of genes with ISRE depends on this factor (6). As presented in Fig. 6C, IRF-1 bound to the ISRE within 1 h following IFN- γ treatment. At that time, the binding of ICSBP was very low, with little recruitment of TEL and HDAC3. By 3 h, however, the levels of ICSBP bound to the ISRE increased by ~10-fold. Accordingly, TEL and HDAC3

recruitment was detectable by this time, and these proteins remained on the ISRE up to 6 h. These results suggest that, during the initial period of IFN- γ treatment (up to ~ 3 h), it is IRF-1 that predominantly binds to the ISRE when TEL and HDAC3 are largely absent from the element. Subsequently, the binding of ICSBP and recruitment of TEL and HDAC3 ensue. Thus, TEL and HDAC3 recruitment and the resultant transcriptional repression may be a postactivation event.

DISCUSSION

By mass-spectrometric analysis we identified TEL, a member of the Ets family, as a protein that is recruited to the ISRE in IFN- γ -stimulated macrophages. TEL recruitment to the ISRE was attributed to the interaction with ICSBP, a member of the IRF family induced by IFN- γ in these cells. TEL recruitment was associated with an increased repression of ISRE-dependent transcription. Providing a plausible mechanism of the increased repression, TEL binding led to the recruitment of HDAC3 to the ISRE in an IFN- γ -dependent manner. Together, our results reveal a novel role for TEL in regulating IFN- γ -dependent transcription, conferred through an interaction with ICSBP.

Mechanism of TEL recruitment. Experiments with recombinant proteins showed that only ICSBP, not IRF-1 or IRF-2, permits TEL to bind to the ISRE, illustrating a specific role for ICSBP in TEL recruitment. Order-of-addition experiments as well as GST pull-down experiments indicated that TEL and ICSBP can associate with each other through a protein-protein interaction. In addition, ICSBP and TEL, when added together, showed an increase in apparent ISRE binding activity, suggesting that the TEL-ICSBP interaction is stabilized by the ISRE.

In our experiments with RAW cell extracts, TEL recruitment was found to be dependent on IFN- γ . This result was interesting, since TEL expression levels were not altered before and after IFN- γ treatment, excluding altered TEL levels as a mechanism of recruitment. A plausible mechanism for IFN- γ -dependent TEL recruitment may include a posttranslational modification of TEL and/or ICSBP, such as phosphorylation. This possibility may be supported by the fact that the TEL gene encodes at least two phosphoproteins, both isoforms being subject to common and distinct phosphorylation events (39). Moreover, ICSBP is shown to be phosphorylated upon IFN- γ treatment (23, 45). On the other hand, it is also possible that TEL recruitment depends on increased expression of ICSBP, which would increase the chance of a protein-protein interaction, even when TEL levels remain constant. These two mechanisms are not mutually exclusive and may contribute to TEL recruitment in concert.

Dual roles of ICSBP. To our knowledge, the interaction between ICSBP and TEL is the second example of the interaction between proteins of the IRF and Ets families. ICSBP has been known to interact with PU.1, a hematopoietic-cell-specific member of the Ets family (4, 5, 38). In addition to ICSBP, PU.1 interacts with Pip, another member of the IRF family, in a phosphorylation-dependent manner (40). Although there is a certain similarity, the ICSBP-TEL interaction differs from the PU.1-ICSBP interaction in several significant ways. First, the PU.1-ICSBP complex binds to the Ets/IRF

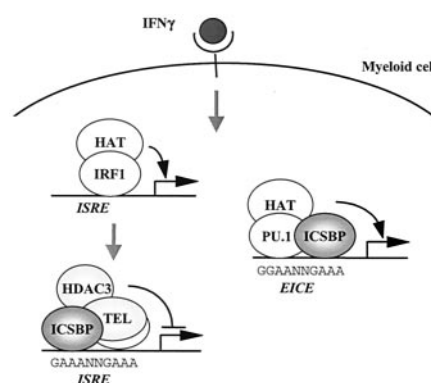


FIG. 7. A model for interaction of ICSBP with TEL. ICSBP interacts with Ets family proteins TEL and PU.1 in IFN- γ -stimulated macrophages. The interaction with TEL occurs on the ISRE, while that with PU.1 requires the EICE. The two partners bind to the elements in opposite orientations: on the ISRE, ICSBP binds to the 5' site and TEL binds to the 3' site, while on the EICE, PU.1 binds the 5' site and ICSBP binds to the 3' site. ICSBP-TEL complex formation may be a late event that occurs following transcriptional activation by IRF-1, as it recruits HDAC3 and represses ISRE-dependent transcription. This repression may be part of a negative-feedback mechanism. On the other hand, the ICSBP-PU.1 complex activates EICE-dependent transcription presumably by recruiting the histone acetylase (HAT) CBP/p300. This model illustrates a dual role of ICSBP that is acquired by the interaction with two different Ets family partners, permitting immune cell-specific gene regulation by IFN- γ .

composite element EICE, consisting of an Ets site (GGAA) and an ISRE half site (GAAA), to which PU.1 and ICSBP bind, respectively (4). However, the ISRE does not have an Ets site to which an Ets family member could bind. Underscoring this difference, PU.1 was not recruited to the ISRE before and after IFN- γ treatment (Fig. 1B). Thus, recruitment of TEL to the ISRE is likely to be strongly dependent on protein-protein interaction. Nevertheless, TEL appears to have some affinity for the ISRE particularly for the 3' GAAA site, as evidenced by the lack of TEL binding to the M4 mutant (Fig. 1C). The possibility that TEL and PU.1 interact with ICSBP in a distinct manner can also be inferred by the difference in the domain requirement for the two proteins. Whereas the ICSBP-TEL interaction requires the DBD of ICSBP, the PU.1-ICSBP interaction is dependent on the IAD domain in the C-terminal region, although the DBD is also required for this interaction (5, 38). Likewise, while the extreme C-terminal region of TEL is required for interacting with ICSBP, PU.1 requires the PEST domain, which is N-terminal to the Ets domain, for interacting with ICSBP (5). That the Ets and IRF proteins bind to the EICE and ISRE in opposite orientations (Fig. 7) may partly account for these differences.

Another notable difference between the ICSBP-TEL interaction and that of PU.1-ICSBP is that they lead to opposite outcomes in transcription, i.e., while the former causes increased repression, the latter results in transcriptional activation. Accumulating evidence indicates that ICSBP and PU.1 activate transcription through EICE and related elements in IFN- γ -stimulated macrophages, as reported for genes encoding the respiratory oxidases gp91^{phox} and p67^{phox} and interleukin-1 β (IL-1 β) (13, 23, 30). ICSBP and PU.1 may also have a role in enhancing expression of the toll-like receptor 4 (42) and

IL-18 (25) through a similar element. This activation seems to involve other factors, notably the coactivator/histone acetylase CBP/p300, with which PU.1 is shown to interact (59). Contrary to the activation by PU.1, TEL led to increased repression of ISRE-dependent gene expression, as evidenced by the reduced reporter activity and reduced 2',5'-OAS expression. In these experiments, where TEL and ICSBP were ectopically expressed, ISRE-dependent transcription was repressed even in the absence of IFN- γ addition, although repression was greater following IFN- γ addition. In view of the existence of a large number of IFN-inducible genes (2), there may be many other genes that are repressed by the two proteins. A microarray-based analysis may provide genome-wide information on target genes regulated by TEL and ICSBP. Combining these results, it appears that ICSBP assumes a dual activity in IFN- γ -stimulated macrophages: on one hand ICSBP and PU.1 cooperatively activate a certain set of genes. On the other hand, by forming a complex with TEL, ICSBP represses gene expression through the ISRE (a model is shown in Fig. 7).

HDAC3 recruitment and biological significance. We found that interaction of ICSBP with TEL results in the recruitment of HDAC3 to the ISRE. In view of the association between histone deacetylases and transcriptional repression documented for a number of promoters (9, 24), the recruitment of HDAC3 is likely to be a mechanism of increased repression. Our results are consistent with the previous report by Wang and Hiebert (55) showing that TEL interacts with HDAC3 and represses Ets-dependent transcription. In that study, TEL was shown to interact with HDAC3 and other corepressors through the domains in the N-terminal region including the PNT domain and a region covering amino acids 268 to 333. Interestingly these regions are outside of the domains required for the interaction with ICSBP (Fig. 3). This nonoverlapping-domain requirement suggests that TEL forms a ternary complex with HDAC3 and ICSBP. Moreover, the observation that the TEL deletion mutants $\Delta 55$ -245 and $\Delta 55$ -332 were unable to repress ISRE promoter activity despite the retention of ICSBP binding activity may now be explained by their inability to recruit HDAC3. It is noteworthy that only HDAC3, not HDAC1 and HDAC2, was recruited to the ISRE. The specific recruitment for HDAC3 is of interest; although class I histone deacetylases HDAC1, HDAC2, and HDAC3 all form multiprotein complexes (20, 51, 61), recent studies indicate that the corepressors N-CoR and SMRT are preferentially assembled into the HDAC3 complex, but not into the HDAC1 or HDAC2 complex, suggesting that HDAC3 has a functional activity distinct from those of other class I histone deacetylases (27, 58). The selective recruitment of HDAC3 may have functional significance for certain promoters in establishing transcriptional repression and alteration of the chromatin environment.

Given that TEL recruitment is dependent on ICSBP, whose expression is increased following IFN- γ treatment (22), TEL recruitment and the resultant transcriptional repression are not likely to be immediate responses to IFN- γ but rather delayed events that follow initial activation of transcription (a model is shown in Fig. 7). In support of this idea, results of kinetics studies shown in Fig. 6C indicated that the binding of IRF-1, a transcriptional activator, precedes the recruitment of TEL and HDAC3, which coincides with ICSBP binding. TEL-mediated repression might be part of a negative-feedback

mechanism designed to restore the basal, low-level transcription and as such may be an integral part of the IFN- γ response. Activation of cytokine signaling is coupled with multiple layers of negative regulation to ensure timely down-regulation of signaling, as exemplified by the SOCS system (17). The TEL-mediated repression may be an example of negative regulation acting at the level of transcription, which may be particularly important for ISRE-carrying genes that are normally stimulated by IFN- α/β (47).

In conclusion, we have described a novel role for TEL in negatively regulating ISRE-dependent gene expression in response to IFN- γ , a role acquired as a consequence of the interaction with ICSBP. Identification of additional genes regulated by TEL and ICSBP may provide further information on a negative-feedback mechanism triggered by IFN- γ .

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T. Kuwata and C. Gongora contributed equally to this work.

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